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Protocols

Detection of major capsid protein of infectious myonecrosis virus in shrimps using monoclonal antibodies

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ABSTRACT

Infectious myonecrosis virus (IMNV) has been causing a progressive disease in farm-reared shrimps in Brazil and Indonesia. Immunodiagnostic methods for IMNV detection, although reliable, are not employed currently because monoclonal antibodies (MAbs) against this virus are not available. In this study, a fragment of the IMNV major capsid protein gene, comprising amino acids 300–527 ($IMNV_{300–527}$), was cloned and expressed in *Escherichia coli*. The nucleotide sequence of the recombinant $IMNV_{300–527}$ fragment displayed a high degree of identity to the major capsid protein of IMNV isolates from Brazil (99%) and Indonesia (98%). Ten MAbs were generated against the expressed fragment, and eight of these, mostly IgG_{2a} or IgG_{2b}, were able to bind to IMNV in tissue extracts from shrimps infected naturally in immunodot-blot assays. Six of these MAbs recognized a ~100 kDa protein in a Western-blot, which is the predicted mass of IMNV major capsid protein, and also bound to viral inclusions present in muscle fibroses and in coagulative myonecrosis, as demonstrated by immunohistochemistry. Among all those MAbs created, four did not cross-react with non-infected shrimp tissues; this observation supports their applicability as a sensitive and specific immunodiagnosis of IMNV infection in shrimps.

1. Introduction

Infectious myonecrosis virus (IMNV) is a non-enveloped virus of the Totiviridae family, containing a 7560 nucleotide long non-segmented double-stranded RNA genome with two open reading frames (ORFs) and an isometric capsid made up of a 901-aa major capsid protein (Poulos et al., 2006; Nibert, 2007; Tang et al., 2008). ORF-1 encodes a putative RNA-binding protein and the major capsid protein, and ORF-2 encodes a putative RNA-dependent RNA polymerase. IMNV infection in cultured penaeid shrimps results in a chronic, persistent, and slowly progressive disease that can reach cumulative mortality rates of up to 70% (Nunes et al., 2004). Typical clinical signs present in infected shrimp are extensive opaque necrotic areas in the striated muscles, particularly in distal abdominal segments and tail fan (Nunes et al., 2004; Lightner et al., 2004; Poulos et al., 2006). Histological injuries are characterized by fluid accumulation in muscle fibers, hemocytic infiltration, fibrosis and basophilic viral inclusions. Epizootic events due to IMNV infection were initially reported in 2002 in a shrimp farm located in the coastal area of Piauí, a state in Northeastern Brazil (Nunes et al., 2004; Lightner et al., 2004; Poulos et al., 2006). Since this first outbreak, IMNV has spread to other states of Northeastern Brazil, where the disease in cultured Pacific white shrimp *Litopenaeus vannamei*, also called *Penaeus vannamei* (Andrade et al., 2007; Pinheiro et al., 2007) was responsible for catastrophic economic losses. By 2006, IMNV outbreaks in shrimp farms had been reported in Indonesia (Senapin et al., 2007), and the unregulated trans-boundary movement of brood stocks and post-larvae for aquaculture was probably the major cause behind the spread of this pathogen to other regions (Flegel, 2006).

Since there is currently no effective treatment for IMNV infection, preventive measures and practices are required for control, and rapid diagnosis is one of the most valid strategies. Current diagnostic methods for the detection of IMNV range from clinical observation and histological examination (Poulos et al., 2006; da Silva et al., 2010) to molecular approaches. Molecular diagnoses include *in situ* hybridization using IMNV-specific gene probes (Tang et al., 2005; Andrade et al., 2008), RT-PCR and nested RT-PCR (Poulos and Lightner, 2006; Senapin et al., 2007), quantitative real-time RT-PCR (Andrade et al., 2007), and reverse-transcriptase loop mediated isothermal amplification reaction (RT-LAMP), followed by nucleic acid detection with a chromatographic dipstick (Puthawibool et al., 2009; Andrade and Lightner, 2009). Although these molecular methods proved to be sensitive and reliable, they

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must be performed in the laboratory by well-trained personnel using specialized equipment and are not suitable for pond-side detection. Simple and reliable methods to assess infection in field conditions such as those available for other shrimp viral diseases, e.g. latex agglutination and immunochromatographic strip tests (Okumura et al., 2005; Powell et al., 2006; Wang and Zhan, 2006; Sithigorngul et al., 2006, 2007), are still lacking for IMNV diagnosis and the development of a practical low-cost and specific diagnosis tests is needed. The present study reports on the use of a recombinant portion of the major capsid protein of IMNV (rIMNV$_{300-527}$) to produce MAbs that react with the native protein in shrimps infected naturally.

2. Materials and methods

2.1. Shrimps and sample preparation

IMNV-infected *L. vannamei* were obtained from commercial shrimp farms in the state of Piauí, Northeastern Brazil, where outbreaks of IMNV occurred in 2007 and 2009. Abdominal muscle samples from shrimps presenting classical clinical signs were dissected and frozen at −80 °C, preserved in RNAlater™ (Qiagen, Hilden, Germany) or Davidson's solution for molecular and immunological approaches. IMNV infection was assessed primarily in shrimps collected in the field by RT-PCR using specific primers to IMNV major capsid protein gene as described below.

2.2. Total RNA isolation and reverse transcription PCR

Preserved shrimp muscle samples were homogenized in Trizol® reagent (Invitrogen, Carlsbad, USA) and total RNA was isolated subsequently following the manufacturer's protocol. RNA concentration and quality were assessed spectrophotometrically at wavelengths 260 and 280 nm (NanoVue™, GE Healthcare, Little Chalfont, UK). First strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse-transcriptase and an oligo(dT)$_{18}$ primer (Invitrogen) at 37 °C for 50 min according to the manufacturer's instructions. Specific PCR to amplify the major capsid protein was performed using Platinum® *Taq* DNA polymerase (Invitrogen) at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, with a final extension step of 72 °C for 5 min in a Mastercycler Gradient termocycler (Eppendorf, Hamburg, Germany). Two specific primers IMNV$_{300-527}$ -F (5′-<u>CTCGAG</u>ACTAAACAAACAACAGACAATGC-3′) and IMNV$_{300-527}$ -R (5′-<u>GGATCC</u>GGAGTCCCATCATATAACTGG-3′) were designed based on the coding sequence for a hydrophilic region of the IMNV major capsid protein, flanking the amino acids 300 to 527 (GenBank accession no. AY570982). The underlined nucleotides in the forward and reverse primers indicate *XhoI* and *BamHI* restriction sites, respectively. The hydrophilic and hydrophobic regions of the IMNV coat protein were predicted by ProtScale software using the Kyte & Doolittle method (http://www.expasy.ch/tools/protscale.html). PCR amplification products were analyzed in 1% agarose gel stained with ethidium bromide, visualized on a Macrovue-UV20 transilluminator (Hoefer, San Francisco, USA) and recorded digitally.

2.3. Cloning, expression, and purification of rIMNV$_{300-527}$

The amplification product of 700 bp was cloned into a pGEM-T Easy vector (Promega, Madison, USA) and transformed into *Escherichia coli* DH5α competent cells (Promega). Six positive recombinant clones were identified by colony PCR and were sequenced in both directions using a MegaBACE 1000 DNA Analysis System (GE Healthcare). All sequences obtained were of high quality (phred ≥15) and both forward and reverse sequences from all six sequenced clones were used to obtain a consensus sequence. Identity of the obtained sequences was determined by similarity search using the BLAST routine from the National Center for Biotechnology Information (NCBI). Deduced amino acid sequence was obtained through Expasy proteomic server and aligned with the capsid protein sequences from both Brazilian (GenBank accession no. AY570982) and Indonesian (GenBank accession no. EF061744) IMNV isolates using the Clustal X software (Thompson et al., 1997).

Subcloning of the IMNV$_{300-527}$ fragment for recombinant protein expression was carried out by excising the fragment with both *XhoI* and *BamHI* enzymes and direct cloning onto pET-14b expression vector (Novagen, Madison, USA) followed by transformation into *E. coli* BL21(DE3) cells (Invitrogen). After growth reached the exponential phase in Luria–Bertani (LB) broth containing 100 μg ml^{-1} of ampicillin, the expression of the recombinant protein rIMNV$_{300-527}$ was induced with 1 mM isopropyl-D-thiogalacto-pyranoside (IPTG, Invitrogen) for 4 h or 12 h at 18 °C, 27 °C, or 37 °C. After centrifugation (5000 × *g* for 10 min at 4 °C), the bacterial pellet was suspended in 20 mM Tris–HCl (pH 7.0), sonicated 3× 30 s at 12 W and then centrifuged at 10,000 × *g* for 30 min at 4 °C. Soluble and insoluble fractions of *E. coli* BL21(DE3) expressing rIMNV$_{300-527}$ were resolved in 12% SDS-PAGE at 20 mA for approximately 2 h, followed by staining with 0.1% Coomassie brilliant blue R-250 for at least 2 h.

With the intent to purify the overexpressed rIMNV$_{300-527}$, which was mainly in the non-soluble fraction, the bacterial pellet was treated with lysis buffer [8 M urea, 100 mM NaH_2PO_4, 100 mM Tris/HCl (pH 8.0)] for 1 h at 60 °C under agitation, followed by centrifugation at 10,000 × *g* for 30 min at 4 °C. The histidine-tagged (His-Tag) rIMNV$_{300-527}$ protein was purified by Ni-NTA Agarose (Qiagen) according the manufacturer's recommendation, with subsequent 4 × 12 h cycles of dialysis in the following buffer: 100 mM Tris–HCl, 500 mM NaCl, 0.5 mM EDTA (pH 8.5), and 10% glycerol. The protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976). Expression and purification efficiency were assessed by SDS-PAGE and further confirmed by Western-blot analysis. Briefly, 12% SDS-PAGE was electro-blotted onto a nitrocellulose (NC) membrane (GE Healthcare), followed by 5% non-fat milk blocking for 90 min, and successive incubations with mouse anti-His-Tag MAb and HRP-conjugated goat anti-mouse IgG (Sigma–Aldrich, St. Louis, USA) for 1 h each. Detection was carried out using the chemiluminescent ECL substrate and radiographic films (GE Healthcare). The films were developed using an automated SRX-101A film processor (Minolta-Konica, Tokyo, USA).

2.4. Monoclonal antibody production

MAbs were prepared according to the procedure described by Kohler and Milstein (1975) and Yokoyama et al. (2006) with some modifications. All procedures involving experimental animals were approved by the UFSC Ethics Committee for Animal Care. Briefly, 6- to 8-week-old Balb/c mice from breeding stocks maintained at the Animal Facility of the Departamento de Microbiologia, Imunologia e Parasitologia, UFSC, were immunized subcutaneously (s.c.) with 50 μg of purified rIMNV$_{300-527}$ mixed with complete Freund's adjuvant (Sigma–Aldrich) at a 1:1 ratio. The mice received the same amount of antigen mixed with incomplete Freund's adjuvant (Sigma–Aldrich) subcutaneously after 10 days. Two additional doses were given at 10-day intervals using the same dose of immunogen by the intraperitoneal route. Four days after the last boost, mice sera were collected by retro-orbital bleeding. The spleens of mice presenting the highest titers against rIMNV$_{300-527}$ were removed under aseptic conditions and dissected, and splenocytes were fused with P3X63Ag8.653 myeloma cells (ATCC® number CRL-1580) at a 5:1 ratio using 50% polyethyleneglycol-

4000 (Sigma–Aldrich) as fusogen. Hybridomas were grown in RPMI-1640 medium (Cultilab, Campinas, Brazil) containing 20% of fetal calf serum (FCS, Cultilab), 1% of antibiotic and antimicotic solution (10,000 U penicillin, 10 ng streptomycin, 25 μg amphotericin B) (Sigma–Aldrich), and supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Sigma–Aldrich). Ten to 14 days after fusion, secreting hybridomas were identified analyzing culture supernatants by ELISA as described below. Selected antibody-producing cultures were cloned two or more times by the limiting dilution method and stored in liquid nitrogen. The immunoglobulin isotype of the MAbs was determined by sandwich ELISA using SBA Clonotyping System/HRP (Southern Biotech, Birmingham, UK), following the manufacturer's instructions.

2.5. Indirect ELISA

The screening and sensitivity evaluation of hybridomas were performed in 96-well plates (Costar, NY, USA) coated for 3 h at 37 °C with 50 ng/well or two-fold serial dilutions ranging from 100 ng to 50 pg/well of rIMNV$_{300–527}$, respectively. Blocking was carried out with 3% bovine serum albumin in PBS containing 0.05% Tween-20 (PBS-T) overnight at 4–8 °C. Hybridoma supernatants were added as primary antibody and incubated at 37 °C for 1 h. Sera of rIMNV$_{300–527}$ immunized mice and cell-free hybridoma culture medium were used as positive and negative controls, respectively. After washing three times with PBS-T, the plates were incubated with HRP-conjugated goat anti-mouse immunoglobulin (Sigma–Aldrich) for 1 h at 37 °C, followed by extensive washing and coloration with O-phenylendiamine (Sigma–Aldrich). Absorbances were determined at 492 nm using a Sunrise^{TM} Basic system (Tecan, Männedorf, Switzerland).

2.6. Reactivity of MAb anti-rIMNV$_{300–527}$ against IMNV-infected tissue homogenate

Frozen striated muscle tissues of IMNV-infected and non-infected shrimps were dissociated in buffer (20 mM Tris, 400 mM NaCl, pH 7.5, v/w) containing a protease inhibitor cocktail (Sigma–Aldrich). After three cycles of sonication at 12 W for 30 s, the homogenates were stored at −20 °C to be used in immunodot- and Western-blot analyses. To determine the sensitivity of the MAbs, 3 μl of purified rIMNV$_{300–527}$ (serially diluted from 90 ng to 75 pg) or tissue homogenates of IMNV-infected or non-infected shrimps (diluted serially from 2 μg to 3 ng) were spotted onto a NC paper and air-dried at room temperature for immunodot-blot analyses. For Western-blot, 90 ng of rIMNV$_{300–527}$ or 3 μg of tissue homogenates of IMNV-infected or non-infected shrimps were resolved in 12% SDS-PAGE and electro-blotted onto NC in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). NC membranes were blocked for 90 min at room temperature with 5% non-fat milk in TBS-T (20 mM Tris, 137 mM NaCl, pH 7.6, containing 0.1% Tween-20), followed by rinses with TBS-T. The membranes were incubated with hybridoma supernatants for 1 h under agitation at room temperature, using supernatant containing a non-correlated MAb against rabies virus as a negative control. Bound antibodies were detected using HRP-conjugated goat anti-mouse immunoglobulin (Sigma–Aldrich). After several washings, chemiluminescent ECL substrate exposure was performed according to the manufacturer's instructions.

2.7. Immunohistochemistry

IMNV-infected and healthy *L. vannamei* were fixed for 2 days with Davidson's alcohol–formalin–acetic acid solution, followed by rinses with 70% ethanol. Paraffin-embedded skeletal tissue was sectioned at 3 μm, and antigen retrieval was performed by immersion of slides for 45 min in 10 mM trisodium citrate buffer (pH 6.0) at 95–98 °C. Tissue sections were incubated overnight at 4 °C with hybridoma supernatants containing MAbs anti-rIMNV$_{300–527}$ or a MAb against rabies virus used as a negative control. After washing with PBS, Envision^{TM} Plus (Dako Cytomation, Glostrup, Denmark) kit, following manufacturer's protocol was used. Slides were also counterstained with Harris's hematoxylin solution, and positive reactions were visualized as a brown color in the cell cytoplasm or in sites of myonecrosis. Images were taken with digital camera (DS-5M-L1; Nikon, Tokyo, Japan) coupled to an Eclipse 50i microscope (Nikon).

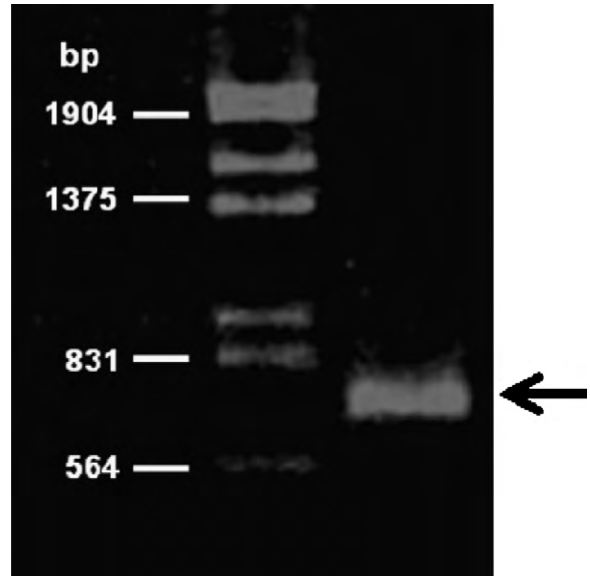

Fig. 1. PCR amplification of a hydrophilic region of IMNV major capsid protein gene (IMNV$_{300–527}$). A 1% agarose gel electrophoresis stained with ethidium bromide showing the PCR amplification product of ~700 bp corresponding to the region 300–527 of the IMNV major capsid protein gene (arrow).

3. Results

3.1. Cloning and sequencing of IMNV$_{300–527}$

IMNV-infected shrimps were collected in 2007 and 2009 from farms in the state of Piauí, Northeastern Brazil, and submitted to total RNA extraction, reverse transcription to cDNA and PCR amplification with specific primers (IMNV$_{300–527}$-F and IMNV$_{300–527}$-R). The primers were directed to a nucleotide sequence encoding a hydrophilic region of the IMNV major capsid protein, encompassing the residues between Thr-300 and Leu-527. The expected PCR product of 700 bp (Fig. 1) was cloned into pGEM-T Easy cloning vector, and six clones had both strands sequenced with high quality (phred ≥15). The nucleotide sequence reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number HM030799. A BLAST similarity search showed that the cloned fragment, named IMNV$_{300–527}$, had 99% and 98% nucleotide identity with the major capsid protein of IMNV isolates from Brazil and Indonesia, respectively. Comparative analysis of the deduced amino acid sequences of these genes fragments by Clustal X indicated that the observed differences are mainly due to non-synonymous substitutions. The described IMNV$_{300–527}$ sequence differs from homologous IMNV sequences from Brazil in positions 356 (V–I) and 515 (R–G) and from Indonesia in positions 356 (V–I), 476 (V–E), 508 (V–I), and 515 (R–G) (Fig. 2).

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                 300                                            346
                  |                                               |
IMNV300-527       TKQTTDNAGNNQSDQIFIHSESTVHIPGQKQMHIVLPRKVNMVNPTT
IMNV Brazil       TKQTTDNAGNNQSDQIFIHSESTVHIPGQKQMHIVLPRKVNMVNPTT
IMNV Indonesia    TKQTTDNAGNNQSDQIFIHSESTVHIPGQKQMHIVLPRKVNMVNPTT

                 347                                            393
                  |                                               |
IMNV300-527       IAEANARVVVQPTYGTVAAGAGVANGNINVAAVGVALPTVNLTDYLV
IMNV Brazil       IAEANARVVIQPTYGTVAAGAGVANGNINVAAVGVALPTVNLTDYLV
IMNV Indonesia    IAEANARVVIQPTYGTVAAGAGVANGNINVAAVGVALPTVNLTDYLV

                 394                                            440
                  |                                               |
IMNV300-527       SWATDFTLGDIKQLVERMKTTLPISRDLMAARQNAMLLSTLFPPLIQ
IMNV Brazil       SWATDFTLGDIKQLVERMKTTLPISRDLMAARQNAMLLSTLFPPLIQ
IMNV Indonesia    SWATDFTLGDIKQLVERMKTTLPISRDLMAARQNAMLLSTLFPPLIQ

                 441                                            487
                  |                                               |
IMNV300-527       SNVASDTKEVPGTAGAYTACLANLGIPETLTVNWGVDINVQPLYQLL
IMNV Brazil       SNVASDTKEVPGTAGAYTACLANLGIPETLTVNWGVDINVQPLYQLL
IMNV Indonesia    SNVASDTKEVPGTAGAYTACLANLGIPETLTVNWGEDINVQPLYQLL

                 488                                    527
                  |                                       |
IMNV300-527       ETDITAHNRYVLNLFKREEVVAGAYEFRWLGHMASYMMGL
IMNV Brazil       ETDITAHNRYVLNLFKREEVVAGAYEFGWLGHMASYMMGL
IMNV Indonesia    ETDITAHNRYVLNLFKREEVIAGAYEFGWLGHMASYMMGL
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Fig. 2. An alignment of the deduced amino acid sequence of $IMNV_{300–527}$ with homologous sequences of IMNV isolates from Brazil and Indonesia. A Clustal X alignment of the $IMNV_{300–527}$ with the major capsid protein sequence of IMNV isolates from Brazil (GenBank accession no. AY570982) and Indonesia (GenBank accession no. EF061744). Amino acid residue differences are highlighted.

3.2. Expression and characterization of $rIMNV_{300–527}$

In order to obtain a recombinant fragment of the IMNV major capsid protein, $IMNV_{300–527}$ was excised from the cloning plasmid and inserted into pET-14b expression vector, resulting in the recombinant plasmid pET-$IMNV_{300–527}$ that was transformed into *E. coli* BL21(DE3) cells. Different culture conditions were tested, mainly culture temperature and time period of induction. The expression of recombinant $IMNV_{300–527}$ ($rIMNV_{300–527}$) was induced by 1 mM IPTG and was found to reach maximum yield after 4 h of induction at 37 °C, although expression could be visualized under all other conditions tested (data not shown). Analysis of both soluble and non-soluble fractions of bacterial cultures expressing $rIMNV_{300–527}$ by SDS-PAGE showed a prominent and exclusive expression of a protein of ∼30 kDa in the non-soluble fraction (Fig. 3a), which was absent in bacteria transformed with mock vector control (Fig. 3a and b). After solubilization of the non-soluble fraction containing $rIMNV_{300–527}$ under protein denaturation conditions, affinity tag protein purification resulted in isolation of pure recombinant protein. Western-blot analysis with an anti-tag antibody confirmed the expression and the identity of the $rIMNV_{300–527}$ (Fig. 3b).

3.3. Production and characterization of MAbs against $rIMNV_{300–527}$

Splenocytes from three mice whose serum showed the highest antibody titers against $rIMNV_{300–527}$ were fused to P3X63Ag8.653 myeloma cells for generating hybridomas. From a total of 910 obtained hybridomas (79% of the initial number), analysis of supernatants by indirect ELISA revealed 30 stable clones reactive against $rIMNV_{300–527}$ in the first screening round (3.3%). Ten out of these 30 clones were selected and submitted to limiting dilutions to reach monoclonality, generating MAbs specific to $rIMNV_{300–527}$, named 1.3G, 1.3H, 1.8C, 2.9C, 2.9E, 3.3A, 9.7F, 9.8D, 9.8H, and 11.2D. Isotyping of these MAbs revealed that the majority of them were IgG2a or IgG2b, except for MAbs 3.3A and 2.9C, which were isotyped as IgG1 and IgM, respectively (Table 1). Immunodot- and Western-blot assays demonstrated that none of the obtained MAbs cross-reacted with *E. coli* protein extracts (data not shown).

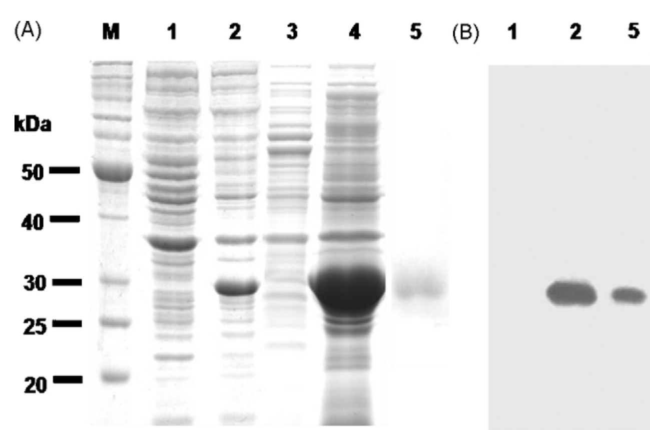

Fig. 3. Heterologous expression and purification of a recombinant fragment of IMNV major capsid protein ($rIMNV_{300–527}$) evaluated by SDS-PAGE and Western-blot. A 12% SDS-PAGE stained with Coomassie blue showing the total protein profile of *E. coli* BL21(DE3) expressing $rIMNV_{300–527}$ (A) and a Western-blot of the same SDS-PAGE as revealed probing with an anti-His-Tag antibody (B). 1 = *E. coli* mock-transfected with empty pET-14b plasmid, 2 = *E. coli* transfected with pET-$IMNV_{300–527}$, induced with IPTG for 4 h at 37 °C, 3 = soluble, 4 = insoluble fractions of *E. coli* expressing $rIMNV_{300–527}$, and 5 = purified $rIMNV_{300–527}$. M = BenchMark™ protein ladder (Invitrogen).

Table 1
Isotyping and reactivity of monoclonal antibodies (MAbs) anti-recombinant and native IMNV major capsid protein in ELISA, immunodot-blot (IDB), Western-blot (WB) and immunohistochemistry (IHC) assays.

MAbs	Isotypes	Reactivity against $rIMNV_{300–527}$		Reactivity against IMNV-infected shrimp tissue		
		ELISA (ng/well)	IDB (pg/spot)	IDB (ng/spot)	WB (band sizes)	IHC
1.3G	IgG2a	~1.56	~375	~5.0	100 kDa	+
1.3H	IgG2a	~3.13	~150	~5.0	–	n.d.
1.8C	IgG2b	~1.56	~95	~10.0	100 kDa	+
2.9C	IgM	<0.05	–	n.d.	n.d.	n.d.
2.9E	IgG2b	~1.56	~95	~22.5	100 kDa	+
3.3A	IgG1	~1.56	~225	~115	100 kDa	+
9.7F	IgG2b	~1.56	~115	–	n.d.	n.d.
9.8D	IgG2a	~3.13	~225	~22.5	80 and 100 kDa	+
9.8H	IgG2b	~1.56	~450	~7.5	–	n.d.
11.2D	IgG2a	~6.25	~225	~255	100 kDa	+

n.d. = not determined.

Sensitivity assays of the obtained MAbs were carried out by ELISA and immunodot-blot using serial dilutions of $rIMNV_{300–527}$ as antigen (Table 1). Most of the MAbs 1.3G, 1.3H, 1.8C, 2.9E, 3.3A, 9.7F, 9.8D, 9.8H, and 11.2D showed similar reactivity when analyzed by ELISA, ranging from 6.25 to 1.56 ng of $rIMNV_{300–527}$. Immunodot-blot tests revealed to be more sensitive than ELISA, where the same MAb panel showed reactivities ranging from 450 to 95 pg of $rIMNV_{300–527}$ immobilized on nitrocellulose membranes. Interestingly, MAb 2.9C was able to react with as little as 50 pg of $rIMNV_{300–527}$ on ELISA, but did not show any binding on an immunodot-blot.

3.4. Detection of IMNV by anti-$rIMNV_{300–527}$ MAbs in shrimp infected naturally

Aiming to identify MAbs able to recognize the native major capsid protein of IMNV, the reactivity of the MAbs anti-$rIMNV_{300–527}$ was evaluated by immunodot-blot, Western-blot, and immunohistochemical assays against skeletal muscle extracts or tissue sections from naturally infected and non-infected *L. vannamei* shrimps. Eight out of nine anti-$rIMNV_{300–527}$ MAbs showed distinct levels of reactivity against IMNV-infected shrimp homogenate by immunodot-blot (Table 1). MAbs 1.3G and 1.3H showed the highest sensitivity among the MAbs tested (5.0 ng/spot), while MAbs 1.8C, 2.9E, 9.8D, and 9.8H showed medium sensitivity (22.5–7.5 ng/spot), and MAbs 3.3A and 11.2D presented the lowest sensitivity, 115 and 225 ng/spot, respectively. MAb 9.7F did not recognize the native major capsid protein in the IMNV-infected shrimp homogenate. For Western-blot assays IMNV-infected and non-infected tissue homogenates were resolved in SDS-PAGE (Fig. 4a), transferred onto nitrocellulose membranes and tested against anti-$rIMNV_{300–527}$ MAbs. Immunoblot reactivity results obtained for MAbs 1.3G, 1.8C, 2.9E, 3.3A, 9.8D, and 11.2D are shown in Table 1 and Fig. 4b, where MAb 1.8C bound specifically to a protein of ~100 kDa in the IMNV-infected shrimp homogenate, as expected. MAbs 1.3H and 9.8H did not recognize the IMNV major capsid protein in infected shrimps by Western-blot and showed non-specific recognition of other proteins. All MAbs showed strong reactivity against $rIMNV_{300–527}$ in immunodot- and Western-blot, as illustrated by MAb 1.8C (Fig. 4b). Despite recognizing the IMNV major capsid protein as did other MAbs, MAb 9.8D also bound non-specifically with a protein of ~80 kDa (data not shown). All other MAbs that recognized the IMNV major capsid protein in tissue homogenates of shrimps infected naturally did not show cross-reactivity against proteins of the non-infected shrimp homogenate (Fig. 4b).

The six MAbs that reacted with the major capsid protein of IMNV in Western-blot were further assayed by immunohistochemistry. All MAbs reacted similarly in myonecrosis sites of abdominal skeletal muscle tissue from IMNV-infected shrimp (Table 1), as illustrated by the results obtained using MAb 1.8C (Fig. 5). Recognition sites marked in brown demonstrated that MAbs were able to bind to IMNV in myonecrosis sites (Fig. 5c, d and e). Immunoreaction was observed in coagulative myonecrosis (Fig. 5d) as well in muscle fibroses, which characterize the chronic stage of IMNV histopathology. MAb 1.8C reacted specifically to viral inclusions present in the cytoplasm of infected cells, as demonstrated in Fig. 5e. In order to investigate the possibility of non-specific recognition of shrimp tissue proteins not associated with IMNV infection, skeletal tissue from non-infected shrimps were assayed against all MAbs, and only two (MAbs 1.3G and 9.8D) showed non-specific binding (data not shown). Four MAbs 1.8C, 2.9E, 3.3A, and 11.2D were highly specific to IMNV, showing no cross-reaction in non-infected shrimp tissue (Fig. 5f), or in infected tissue exposed to non-related anti-rabies virus MAb (Fig. 5b).

4. Discussion

In the present report, production of anti-IMNV MAbs was based on mice immunization with a recombinant portion of the major capsid protein of IMNV ($rIMNV_{300–527}$) expressed in a prokaryotic system. Previous reports have also described heterologous

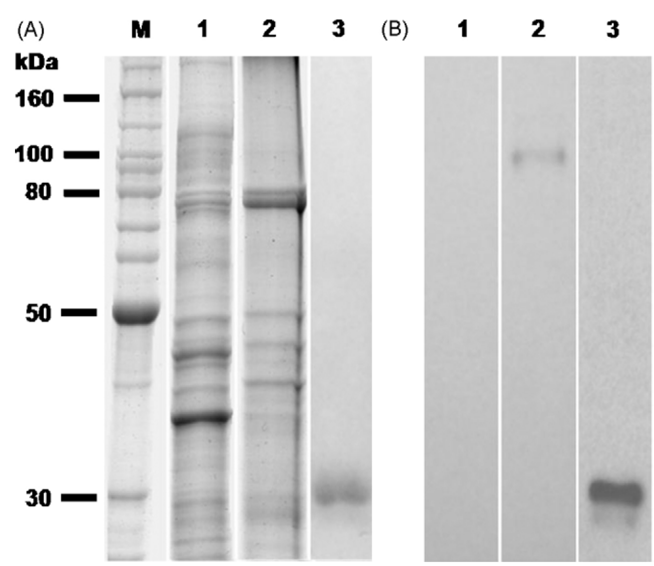

Fig. 4. Detection of native IMNV major capsid protein in tissue homogenate of *Litopenaeus vannamei* by Western-blot using the anti-$rIMNV_{300–527}$ MAb 1.8C. A 12% SDS-PAGE stained with Coomassie blue (A) and a Western-blot of the same gel (B) revealed using the MAb 1.8C (IgG2b). 1 = striated muscle homogenate from non-infected shrimp; 2 = striated muscle homogenate from IMNV-infected shrimp; 3 = $rIMNV_{300–527}$. M = BenchMark^{TM} protein ladder (Invitrogen).

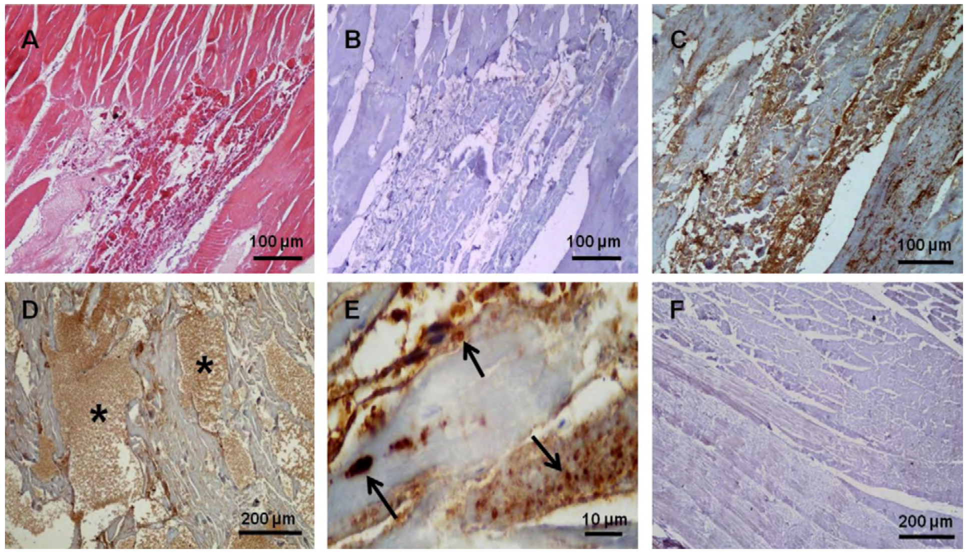

Fig. 5. Immunohistochemical analyses of abdominal skeletal tissue from IMNV-infected *Litopenaeus vannamei* using MAb 1.8C. IMNV-infected (A, B, C, D and E) and non-infected (F) abdominal skeletal tissues were stained with H&E (A) or incubated with hybridoma culture supernatants containing MAb 1.8C (C, D, E and F) or a non-correlated MAb against rabies virus as negative control (B), followed by HRP-conjugated antibody. Slides were colored with 3,3-diaminobenzidine and counterstained with Harris's hematoxylin solution. Arrows indicate viral inclusions and asterisk shows coagulative myonecrosis.

systems for the expression of viral proteins for the production of MAbs (Sithigorngul et al., 2009; Chaivisuthangkura et al., 2010a,b). Heterologous systems help to acquire enriched preparations of proteins that are either difficult to obtain or present at low copy numbers in the natural hosts. The rIMNV$_{300-527}$ encompasses the amino acids Thr-300 to Leu-527. This fragment was chosen due the presence of hydrophilic regions within the major capsid protein of IMNV as well as epitopes that may be potentially exposed on the virion surface, thus facilitating virus recognition by MAbs.

Nucleotide sequences of IMNV$_{300-527}$, a gene fragment originated from a Brazilian isolate, were obtained by DNA sequencing and analysis of both the nucleotide and predicted amino acidic sequences confirmed the identity of this fragment as an IMNV protein. The deduced amino acid sequence of IMNV$_{300-527}$ showed a close relationship with IMNV sequences from Brazil and Indonesia reported previously (Poulos et al., 2006; Senapin et al., 2007). The 1–2% of divergence between them is due to non-synonymous substitutions of four amino acids. Two of these four amino acids are similar to the other Brazilian sequence but diverged from Indonesian isolate, probably because of a genetic variation caused by geographic isolation, a phenomenon already observed for shrimp viruses (Marks et al., 2004; Senapin et al., 2007). Genotype variation has also been reported for shrimp virus from the same geographical area (Wongteerasupaya et al., 2003; Tan et al., 2009), and might be the reason of the two exclusive substitutions of IMNV$_{300-527}$ sequence in comparison to IMNV isolates described previously. However, despite these few variations, the high identity between rIMNV$_{300-527}$ and the IMNV isolates from Brazil and Indonesia (98–99% amino acid identities) suggests that the epitopes in this portion of the capsid protein are ubiquitous among different virus isolates. Thus, the MAbs elicited against rIMNV$_{300-527}$ should be applicable for detection of IMNV in monitoring and control programs worldwide.

Ten MAbs generated in the present study showed different sensitivities against rIMNV$_{300-527}$ on ELISA and immunodot-blot. MAb sensitivities through immunodot-blot analyses using recombinant proteins of other shrimp viruses have also been reported, and results were very similar to the findings described above: the sensitivities ranged between 70 and 800 pg of the protein (Sithigorngul et al., 2009; Chaivisuthangkura et al., 2010a,b). Moreover, among all five specific MAbs identified in Western-blot, four showed high specificity to viral replication sites in areas of muscle fibroses, suggesting that those MAbs are excellent tools for future studies of cytolocalization and viral distribution among different shrimp organs.

Although MAb 1.3G did not show cross-reactivity with non-IMNV proteins in Western-blot, it was able to bind to healthy shrimp tissue; the difference in binding is probably due to epitope changes during fixation. Among all immunoassays employed in IMNV detection, the interaction of most MAbs with their ligands was not affected by conformation changes of the epitope, since they showed a very strong reaction with native antigens in immunodot-blot, as well as denatured antigens in Western-blot and immunohistochemical analysis. Similar results were demonstrated in other reports that evaluated the reactivity of MAbs generated against Taura syndrome virus (TSV), *Penaeus stylirostris* densovirus and white spot syndrome virus (WSSV) using the same techniques (Sithigorngul et al., 2009; Chaivisuthangkura et al., 2010a,b).

Besides *L. vannamei*, an organism found infected naturally with IMNV, *L. stylirostris* (also called *Penaeus stylitostris*), *Penaeus monodon* and *Farfantepenaeus subtiltis* (also called *P. subtiltis*), have been shown susceptible to experimental infection (Tang et al., 2005; Coelho et al., 2009). Several reports have demonstrated the diversity of reservoir hosts in terms of other shrimp viruses, such as WSSV and TSV that can infect or be transmitted by many cultured and wild crustaceans in addition to shrimp (Lo et al., 1996; Overstreet et al., 1997; Kanchanaphum et al., 1998; Chen et al., 2000; Chapman et al., 2004; Kiatpathomchai et al., 2008). Further studies to evaluate the IMNV reservoir hosts are necessary in order to elucidate the IMNV transmission chain, and the MAbs described herein might be useful in the identification of such reservoir hosts among shrimps and other animal species.

In conclusion, the present report describes the development of four MAbs highly specific to IMNV, since they did not react with healthy shrimp proteins in immunodot-blot, Western-blot, and immunohistochemical assays. Although further studies are necessary to investigate the absence of cross-reactivity of those MAbs with other shrimp viruses, they are very versatile in their ability to adapt to several immunological tests formats and can be considered promising tools for the development of rapid and simple immunoassays, such as immunochromatographic strip tests. A mixture of two MAbs could be used in the development of a commercial detection kit, increasing the sensitivity of the method and leading to a detection system that can be easily used in a non-laboratory environment, providing opportunities for limiting the extent of viral spread in farms infected with IMNV.

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